NOVEL POLYMERASE COMPOSITIONS AND USES THEREOF

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Related Applications

This application is a continuation-in-part of co-pending U.S. patent application 08/164,290 which was filed December 8, 1993.

Field of the Invention

The present invention is related to the field of molecular biology, and more particularly, to polynucleotide synthesis.

BACKGROUND OF THE INVENTION

DNA polymerases catalyze the synthesis of DNA and can be found in all cells as well as being encoded in numerous viruses. Although all DNA polymerases possess 5'-3' DNA polymerase activity, DNA polymerases differ from one another in numerous other properties. For example, some enzymatic activities that are possessed by some DNA polymerases, but absent in other DNA polymerases include: double stranded DNA 5'-3' exonuclease activity, single-stranded DNA 3'-5' exonuclease activity, double-stranded 3'-5' DNA exonuclease activity, RNase H activity, reverse transcriptase activity, and the like. Additionally, different DNA polymerases may have different optimal pH and temperature ranges for activity. Furthermore, DNA polymerases may differ in the rate in which they catalyze DNA synthesis.

Purified DNA polymerases have numerous uses in vitro. A detailed description of DNA polymerases, including methods for their isolation, can be found among other places, in DNA Replication 2nd edition, by Kornberg and Baker, W.H. Freeman & Company, New York, New York 1991. In vitro uses of DNA polymerases include, for example, the labeling and synthesis of hybridization probes, DNA sequencing, and DNA amplification. A DNA amplification method employing DNA polymerases that has been particularly useful is the polymerase chain reaction (PCR) technique. The

technique of PCR is described in numerous publications, including, PCR: A Practical Approach, M.J. McPherson, et al., IRL Press (1991), PCR Protocols: A Guide to Methods and Applications, by Innis, et al., Academic Press (1990), and PCR Technology: Principals and Applications for DNA Amplification, H.A. Erlich, Stockton Press (1989). PCR is also described in many U.S. Patents, including U.S. patents, 4,683,195; 4,683,202; 4,800,159; 4,965,188; 4,889,818; 5,075,216; 5,079,352; 5,104,792; 5,023,171; 5,091,310; and 5,066,584, which are hereby incorporated by reference. The PCR technique typically involves the step of denaturing a polynucleotide, followed by the step of annealing at least a pair of primer oligonucleotides to the denatured polynucleotide, i.e., hybridizing the primer to the denatured polynucleotide template. After the annealing step, an enzyme with polymerase activity catalyzes synthesis of a new polynucleotide strand that incorporates the primer oligonucleotide and uses the original denatured polynucleotide as a synthesis template.

In many instances, a given DNA polymerase may fail to synthesize the desired polynucleotide product. These failures may be attributable to a number of reasons including such problems as template and primer base pair mismatches, lack of proofreading, insufficient rate of synthesis, high misincorporation rate, inability to transcribe GC or AT rich regions, lack of sufficient processivity (processivity refers to the length of synthesis product formed before the polymerase stops synthesis), etc. It is therefore of interest to provide new methods and compositions for improved polynucleotide synthesis over a wide variety of experimental conditions.

SUMMARY OF THE INVENTION

The subject invention provides novel compositions containing a mixture of (a) an enzyme that possesses substantial 3'-5' exonuclease activity and (b) a DNA polymerase with less 3'-5' exonuclease activity than the enzyme possessing substantial 3'-5' exonuclease activity. Preferably, the enzyme with substantial 3'-5' exonuclease activity is a DNA polymerase. Preferably, the DNA polymerase with less 3'-5' exonuclease activity than the enzyme possessing substantial 3'-5' exonuclease activity is a DNA polymerase substantially lacking 3'-5' exonuclease activity. When a step in a technique

of interest employing polynucleotide synthesis involves the step of incubation at an elevated temperature, e.g., PCR, both the DNA polymerase and the enzyme with substantial 3'-5' exonuclease activity are thermostable enzymes. A preferred embodiment of the invention is a composition comprising the *Taq* DNA polymerase (from Thermus aquaticus) and the *Pfu* DNA polymerase (from Pyrococcus furiosus).

Another aspect of the invention is to provide methods for synthesizing polynucleotides, typically DNA, using compositions comprising an enzyme that possesses substantial 3'-5' exonuclease activity and DNA polymerase with less 3'-5' exonuclease activity than the enzyme with substantial 3'-5' exonuclease activity. Preferably, the DNA polymerase used in the provided methods is a DNA polymerase that substantially lacks 3'-5' exonuclease activity. The methods of synthesizing DNA (or other polynucleotides) provided comprise the step of mixing a composition containing (a) an enzyme possessing substantial 3'-5' exonuclease activity and (b) a DNA polymerase with less 3'-5' exonuclease activity than the enzyme with substantial 3'-5' exonuclease activity. Other reagents required for polynucleotide synthesis include nucleotide triphosphates (dNTPs), polynucleotide primers, a synthesis template, and the like.

Another aspect of the invention is to use the subject method of polynucleotide synthesis to carry out the synthesis step in a polymerase chain reaction experiment.

Yet another aspect of the invention is to provide kits for the synthesis of polynucleotides, wherein the kits comprise an enzyme that possesses substantial 3'-5' exonuclease activity and a DNA polymerase with less 3'-5' exonuclease activity than the enzyme with substantial 3'-5' exonuclease activity, preferably a DNA polymerase substantially lacking 3'-5' exonuclease activity. Preferably, the enzyme with substantial 3'-5' exonuclease activity is a DNA polymerase. The kits may also contain polynucleotide precursors, synthesis primers, synthesis templates, buffers, and the like.

DESCRIPTION OF SPECIFIC EMBODIMENTS

The subject invention provides novel composition for use in synthesizing polynucleotides, particularly DNA. The subject compositions comprise an enzyme that possesses substantial 3'-5' exonuclease activity and a DNA polymerase with less 3'-5'

exonuclease activity than the enzyme with substantial 3'-5' exonuclease activity. In a preferred embodiment of the invention, the DNA polymerase in the compositions is a DNA polymerase that substantially lacks 3'-5' exonuclease activity. The enzyme that possesses substantial 3'-5' exonuclease activity is preferably a DNA polymerase. Prior to the inventors' work, DNA synthesis in vitro was performed with a single purified DNA polymerase. In a variety of synthesis procedures, the subject compositions provide superior synthesis results, as compared with the synthesis results obtained with a single DNA polymerase with less 3'-5' exonuclease activity than the enzyme with 3'-5' exonuclease activity alone (including synthesis results obtained with DNA polymerases that substantially lack 3'-5' exonuclease activity).

Although compositions comprising a DNA polymerase with less 3'-5' exonuclease activity than the enzyme possessing substantial 3'-5' exonuclease activity may produce superior results in a variety of synthesis experiments, the composition is especially useful in DNA synthesis when there exists one or more mismatched nucleotide(s), particularly mismatches at the 3' end of one or more synthesis primer(s). In such situations, the results achieved, i.e., the amount of synthesis product produced, are significantly greater than the amount of synthesis product obtained using either a DNA polymerase with less 3'-5' exonuclease activity than the enzyme possessing substantial 3'-5' exonuclease activity or with a DNA polymerase possessing substantial 3'-5' exonuclease activity alone. Other advantages of the subject compositions and methods include increased synthesis product yield, increased transcription product length, and the synthesis of polynucleotides that can not be synthesized by a given DNA polymerase alone.

The enzyme possessing substantial 3'-5' exonuclease activity for use in the subject compositions and methods may be any enzyme possessing substantial 3'-5' single- stranded DNA exonuclease activity. Enzymes possessing substantial 3'-5' exonuclease activity for use in the present compositions and methods may be isolated from natural sources or produced through recombinant DNA techniques. Enzymes that possess substantial 3'-5' exonuclease activity and their properties are described in detail in, among other places, DNA Replication 2nd edition, Kornberg and Baker, supra and Enzymes, supra. Examples of enzymes that possess substantial 3'-5' exonuclease

activity include E. coli exonuclease I, E. coli exonuclease III, E. coli recBCD nuclease, mung bean nuclease, and the like. Preferred enzymes with substantial 3'-5' exonuclease activity for use in the subject compositions and methods are DNA polymerases that possess substantial 3'-5' exonuclease activity.

DNA polymerases that possess substantial 3'-5' exonuclease activity include the *Pfu* DNA polymerase, E. coli DNA polymerase I, Klenow fragment, T-4 polymerase, T-7 polymerase, E. coli DNA pol III, Ultima DNA Polymerase (Cetus), Vent DNA and Deep Vent DNA polymerases (New England Biolabs). When using the subject compositions in reaction mixtures that are exposed to elevated temperatures, e.g., during the PCR technique, use of thermostable DNA polymerases is preferred. Examples of the thermostable DNA polymerases that possess substantial 3'-5' exonuclease activity include Vent DNA polymerase, Ultima DNA polymerase, Deep Vent DNA polymerase, and *Pfu* DNA polymerases. A particularly preferred DNA polymerase possessing 3'-5' exonuclease activity for use in subject composition is the *Pfu* DNA polymerase. The *Pfu* DNA polymerase is commercially available from Stratagene (La Jolla, California). A detailed description of the *Pfu* DNA polymerase can be found, among other places in U.S. Patent Application serial number 07/803,627 filed December 2, 1991.

DNA polymerases and their properties are described in detail in, among other places, DNA Replication 2nd edition, Kornberg and Baker, W.H. Freeman, New York, New York (1991). DNA polymerases with less 3'-5' exonuclease activity for use in the subject compositions and methods may be isolated from natural sources or produced through recombinant DNA techniques. DNA polymerases with less 3'-5' exonuclease activity than the 3'-5' exonuclease activity of the enzyme with substantial 3'-5' exonuclease activity, i.e., 3'-5' single-stranded exonuclease activity, include *Taq* DNA polymerases and Sequenase™ (modified bacteriophage T7 DNA polymerase, available from U.S. Biochemical, Columbus, Ohio), and the like. Additionally, the person of average skill in the art having the benefit of this disclosure will recognize that exonuclease deficient polymerases such as Exo⁻ *Pfu* DNA polymerase, Vent® (exo⁻) DNA polymerase, Deep Vent® (exo-) DNA polymerase, and the like may be suitably used in the subject compositions. *Taq* DNA polymerase, Sequenase™, Exo⁻ *Pfu* DNA

polymerase, Vent (exo$^-$) DNA polymerase, and Deep Vent (exo$^-$) DNA polymerase are all examples of DNA polymerases that substantially lack 3'-5' exonuclease activity.

When using the subject compositions in reaction mixtures that are exposed to elevated temperatures, e.g., during the PCR technique, use of thermostable DNA polymerases is preferred. The subject composition may also be used with DNA polymerases that have not yet been isolated, provided that the DNA polymerases have less 3'-5' single-stranded DNA exonuclease activity than the enzyme with substantial 3'-5' exonuclease activity in the subject composition. Assays for both DNA polymerase activity and 3'-5' exonuclease activity can be found in DNA Replication 2nd Ed., Kornberg and Baker, supra, Enzymes, Dixon and Webb, Academic Press, San Diego, CA (1979), as well as other publications available to the person of ordinary skill in the art. A preferred DNA polymerase for use in the subject compositions and methods of the invention is the *Taq* DNA polymerase.

The term "substantially lacking 3'-5' exonuclease activity" when used in reference to a DNA polymerase, refers to those DNA polymerases that have less 3'-5' exonuclease activity than the enzyme included in the subject composition that has substantial 3'-5' exonuclease activity.

The term "thermostable" when used with respect to an enzyme, is readily understood by a person of ordinary skill in the art. Typically, a "thermostable" enzyme retains at least 50 percent of its specific activity after exposure to a temperature of 80°C for a period of 20 minutes.

The ratio of the DNA polymerase with less 3'-5' exonuclease activity than the enzyme possessing substantial 3'-5' exonuclease activity to the enzyme possessing substantial 3'-5' exonuclease activity in the subject composition may be varied with respect to one another. The ratio of the DNA polymerase activity to 3'-5' exonuclease activity present in the subject composition employed in a given synthesis procedure may be readily optimized by performing a series of simple experiments in which the ratio of the DNA polymerase with less 3'-5' exonuclease activity than the enzyme with substantial 3'-5' exonuclease activity to the enzyme possessing substantial 3'-5' exonuclease activity are systematically varied with respect to one another and the synthesis results compared.

The subject compositions may be used in various methods of synthesizing polynucleotides in essentially the same manner as the DNA polymerase present in the subject composition. Typically, synthesis of a polynucleotide requires a synthesis primer, a synthesis template, polynucleotide precursors for incorporation into the newly synthesized polynucleotide, (e.g. dATP, dCTP, dGTP, dTTP), and the like. Detailed methods for carrying out polynucleotide synthesis are well known to the person of ordinary skill in the art and can be found, for example, in Molecular Cloning second edition, Sambrook et al., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989). The process of PCR employs a polynucleotide synthesis step in each cycle; this polynucleotide synthesis step may be achieved using the subject compositions.

The invention herein also contemplates a kit format which comprises a package unit having one or more containers of the subject composition and in some embodiments including containers of various reagents used for polynucleotide synthesis, including synthesis in PCR. The kit may also contain one or more of the following items: polymerization enzymes, polynucleotide precursors, primers, buffers, instructions, and controls. Kits may include containers of reagents mixed together in suitable proportions for performing the methods in accordance with the invention. Reagent containers preferably contain reagents in unit quantities that obviate measuring steps when performing the subject methods.

The following examples are offered for the purpose of illustrating, not limiting, the subject invention.

Background of Examples

CHARACTERISTICS OF *TAQ* AND *PFU* DNA POLYMERASES

Thermus aquaticus DNA Polymerase

Thermus aquaticus (*Taq*) DNA polymerase is a 94-kDa protein which does not have an inherent 3' to 5' exonuclease activity (Tindall, K.R., and T.A. Kunkel. 1988. Fidelity of DNA synthesis by the *Thermus aquaticus* DNA polymerase. *Biochemistry* **27**:6008-6013). 3' to 5' exonuclease activity enables a polymerase to proofread and is

therefore associated with fidelity of an enzyme. The estimated error rate of *Taq* varies from 2 x 10^{-4} mutations per nucleotide per cycle during PCR (Saiki, R.K., D.H. Gelfand, S. Stoffel, S.J. Scharf, R. Higuchi, G.T. Horn, K.B. Mullis, and H.A. Erlich. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. 1988. *Science* **239**:487-491) to 2 x 10^{-5} for nucleotide substitution errors in a single round of DNA synthesis of the *lacZα* gene (Eckert, K.A. and T.A. Kunkel. High fidelity DNA synthesis by the *Thermus aquaticus* DNA polymerase. 1990. *Nucleic Acids Res.* **18**:3739-3744). The error rate of *Taq* DNA polymerase is important in polymerization because it reflects the ability of the polymerase to extend from a mismatched primer:template. *Taq* DNA polymerase has been shown to extend significantly less efficiently from a mismatched primer: template than from a correctly based paired primer:template (Innis, M.A., K.B. Myambo, D.H. Gelfand and M.A.D. Brow. DNA sequencing with *Thermus aquaticus* DNA polymerase and direct sequencing of polymerase chain reaction-amplification of DNA. 1988. *Proc. Natl. Acad. Sci. USA.* **85**:9436-9440 and Kwok, S., D.E. Kellogg, D. Spasic, L. Goda, C. Levenson, and J.J. Sninsky. Effects of primer-template mismatches on the polymerase chain reaction: Human immunodeficiency virus type 1 model studies. 1990. *Nucleic Acids Res.* **18**:999-1005).

Taq DNA polymerase is highly processive at an extension rate of >60 nucleotides per second at 70°C on M13 phage DNA when using a GC-rich 30 mer as a primer (Innis, et al. 1988). The *Taq* genome is 66.8% GC (Munster, M.J., A.P. Munster, J.R. Woodrow, and R.J. Sharp. Isolation and preliminary taxonomic studies of *Thermus* strains isolated from Yellowstone National Park, USA. 1986. *J. of Gen. Microbiol.* **132**:1677-1683).

Pyrococcus furiosus DNA polymerase

Pyrococcus furiosus (*Pfu*) DNA polymerase is a 91-kDa protein which has an inherent 3' to 5' exonuclease activity. This proofreading activity allows *Pfu* to extend from mismatched primer:templates by first removing the mismatched base(s) followed by polymerization and results in an error rate of 1.6 x 10^{-6} mutations per nucleotide per cycle in PCR reactions. The error rate of *Pfu* DNA polymerase is thus tenfold lower

than that of *Taq* DNA polymerase and results in higher fidelity (Lundberg, K.S., D.D. Shoemaker, M.W.W. Adams, J.M. Short, J.A. Sorge, and E.J. Mathur. High-fidelity amplification using a thermostable DNA polymerase isolated from *Pyrococcus furiosus*. 1991. *Gene* **108**:1-6). The processivity of *Pfu* DNA polymerase is 10-15 nucleotides per second and its genome is 38% GC.

TEMPLATES

cDNA templates

cDNAs from 2 sources, hybridoma and PBLs (peripheral blood lymphocytes), were chosen as templates in these experiments. Hybridoma cDNA from human anti-tetanus toxoid 9F12 (ATCC, HM-8177) and mouse anti-human fibronectin CG7C7 (ATCC, HB-126) express only one immunoglobulin heavy and light chain and therefore would contain a homogeneous population of heavy and light chain cDNAs. The 9F12 cDNA had amplified efficiently in previous experiments when using primers AB-61 or MK-205 with AB-76 (Fd) or MK-39 (VH), AB-25 and AB-94 (light chain[LC]), and MK-94 and AB-76 (CH1). Based on nucleotide sequence, 9F12 is a human IgG1 from the VHIII family with a *kappa* light chain. The SA-13 cDNA had amplified efficiently in previous experiments when using primers AB-19 with AB-41 (Fd) and AB-25 and AB-26 (LC). PBL cDNA expresses immunoglobulin heavy and light chains from all of the human heavy and light chain families (Kabat, T.T. Wu, H. Bilofsky, M. Reid-Milner, and H. Perry, eds. Sequences of Proteins of Immunological Interest, 1987, U.S. Public Health Service, Washington, D.C.) and therefore would contain a heterogenous population of heavy and light chain cDNAs. This cDNA had not amplified efficiently with primer AB-61 or MK-205 with AB-76 (Fd) or MK-39 (VH) but had amplified efficiency with AB-25 and AB-94 (LC) and MK-94 and AB-76 (CH1) (Table 13).

DNA templates

Nine different DNA templates were also used in these experiments. The genomic DNAs were isolated from human, Epstein Barr virus, *Escherichia coli*, and transgenic and nontransgenic mouse. Plasmid DNAs were pBluescript II and pBluescript containing the light chain and Fd of an anti-tetanus toxoid immunoglobulin

(Mullinax R.L., E.A. Gross, J.R. Amberg, B.N. Hay, H.H. Hogrefe, M.M. Kubitz, A. Greener, M. Alting-Mees, D. Ardourel, J.M. Short, J.A. Sorge, and B. Shopes. 1990. Identification of human antibody fragment clones specific for tetanus toxoid in a bacteriophage lambda immunoexpression library. *Proc. Natl. Acad. Sci. USA* **87**:8095-8099).

PRIMER DESIGN

The ability of an oligonucleotide to act as a primer in DNA synthesis is dependent on several factors: the kinetics of association and dissociation of the primer:template duplexes under the annealing and extension conditions, the effects of mismatched bases and their location on duplex stability, and the efficiency with which the polymerase can recognize and extend from a mismatched duplex. In general, single base pair mismatches at or near the terminal 3' base of a primer are known to affect the ability of the polymerase to bind and extend from the primer:template and therefore should have a significant effect on the efficiency of the priming reaction.

In particular, *Taq* DNA polymerase requires that 3 to 5 bases at the 3' end of the primer base pair exactly in order for polymerization to occur. The effect of other mismatches on the efficiency of polymerization is dependent on the number of mismatches and where they occur in the primer. This presents a problem when the exact template sequence is not known such as when the nucleotide sequence of the template is derived from amino acid sequence due to the redundancy of the amino acid code and when designing primers for templates of families of genes which are heterogeneous.

Four oligonucleotide primers designed to specifically prime the first constant region of human heavy chain immunoglobulin genes (VH) were designed based on the available nucleotide and amino acid sequences with the problems above in mind (Mullinax, et al., 1990). Because experimental results indicated poor priming and/or amplification efficiency with PBL templates with *Taq* DNA polymerase, we wanted to investigate how this efficiency could be improved.

One of the human heavy chain first constant region primers, AB-61 (Table 15) was chosen for examination. AB-61 has a dGTP as the 3' base and would require a

dCTP in the corresponding position in the template in order for efficient priming to occur when *Taq* DNA polymerase was used for primer extension due to its lack of 3' to 5' exonuclease activity (Kwok, et al. 1990, supra). However, *Pfu* DNA polymerase does have 3' to 5' exonuclease activity and would remove any mismatched base(s) from the 3' end of the primer and would therefore be able to extend (Lundberg, et al. 1991, supra). Therefore it was of interest to investigate the effect of using *Pfu* DNA polymerase alone and in combination with *Taq* DNA polymerase in primer extension reactions using cDNA from hybridoma cell lines (9F12 and CG7C7) and from human PBLs as the template under various experimental conditions.

Kwok, et al. (1990) demonstrated that primer extension efficiency when using *Taq* DNA polymerase is independent of the dNTP in the template when a dTTP is the 3' base in the primer. Therefore the effect of the addition of one or more dTTPs in various positions at the 3' end of the primer AB-61 (Table 13) was also investigated.

BUFFER COMPARISONS

Buffer conditions have been shown to effect the processivity, activity and fidelity of polymerases. In particular, the processivity and/or activity of *Taq* DNA polymerase are known to be affected by KCl, $MgCl_2$, $(NH_4)_2SO_4$, and NaCl concentrations. The fidelity of *Taq* DNA polymerase is also affected by the concentration of $MgCl_2$ relative to the total concentration of dNTPs (Eckert, et al. 1990, supra) and dNTP and MnCl2 concentrations (Leung, D.W., E. Chen, and D.V. Goeddel. 1989). A method for random mutagenesis of a defined DNA segment using a modified polymerase chain reaction. *Technique*. **1**:11-15).

Table 17 lists the buffer components and their concentrations for the 10x *Taq* DNA polymerase and *Pfu* V25, #1, and #3 buffers used in these experiments.

DIFFERENT RATIOS OF *TAQ* AND *PFU* DNA POLYMERASES

Taq DNA polymerase is unable to correct nucleotide misincorporations made during polymerization due to its lack of 3' to 5' exonuclease activity. In general, this would result in the inability of *Taq* to extend from a newly polymerized strand annealed to a template when an incorrect nucleotide has been inserted. *Pfu* DNA polymerase has

an inherent 3' to 5' exonuclease activity and would be able to remove the incorrectly inserted nucleotide and then extend from the correctly base paired primer:template. The ratio of the two polymerases may be critical for optimal efficiency of this process. We therefore compared several ratios of *Taq:Pfu* DNA polymerases to determine the effect on DNA synthesis from 3' matched and mis-matched primers.

In addition, *Pfu* DNA polymerase has been shown to degrade unannealed primers by its 3' to 5' exonuclease activity. These degraded primers would not be available in subsequent rounds of DNA amplification and would therefore effect the efficiency of the PCR reaction. It may therefore be desirable to have a low concentration of *Pfu* DNA polymerase relative to *Taq* DNA polymerase to decrease this effect.

EXAMPLE 1

PRIMER EXTENSION WITH *TAQ* OR *PFU* DNA POLYMERASE IN SEPARATE REACTIONS

These experiments demonstrate the relative ability of *Taq* or *Pfu* DNA polymerase to PCR amplify hybridoma and PBL templates under various conditions. *Taq* DNA polymerase resulted in either the presence of a PCR product or an increase in the amount of PCR product when compared to *Pfu* DNA polymerase when amplifying hybridoma and PBL templates. In addition, the dNTP and not the $MgCl_2$ concentration affected the amount of LC product generated.

Taq and *Pfu* DNA polymerases with CG7C7 hybridoma template

Taq and *Pfu* DNA polymerases were compared for their ability to amplify the LC, CH1, and Fd regions of a mouse anti-human fibronectin antibody (CG7C7, ATCC HB-126). Total RNA was isolated from CG7C7 hybridoma cells using an RNA isolation kit (Stratagene). Five µg of total RNA was converted to cDNA in a first strand synthesis reaction using an oligo-dT primer for the light chain and AB-41 for the heavy chain (Table 15). mRNA was annealed to the first strand primer at 65°C for 5 minutes followed by cooling at room temperature for 30 minutes. First strand reactions were performed in 1x first strand buffer [1x first strand buffer is 75 mM KCl; 50 mM tris-Cl, pH 8.3; 10 mM dithiothreitol; 3 mM $MgCl_2$; 1 Unit RNase Block II

(Stratagene)], 375 μM each dNTP and 20 Units Moloney-Murine Leukemia Virus Reverse Transcriptase (M-MLVRT) (Stratagene)] and incubated at 37°C for 1 hour and then 42°C for 30 minutes.

PCR primers used to amplify the LC were AB-25 and AB-26, CH1 were MK-501 and AB-41, Fd were AB-41 and AB-19, and the polylinker region of pBluescript were T3 and T7 (Table 14). PCR reactions with *Taq* DNA polymerase were at a primer concentration of 0.2 μM of each 5' and 3' primer in 1x *Taq* buffer (Table 17) with 200 μM each dNTP and 2.5 units *Taq* DNA polymerase (Stratagene; La Jolla, California) using 1/10 of cDNA prepared in the first strand reaction as the template. Samples were denatured at 99°C for 5 minutes and annealed at 54°C for 5 minutes followed by PCR amplification at 72°C for 3 minutes, 93°C for 1.5 minutes and 54°C for 2 minutes for 30 cycles.

PCR reactions with *Pfu* DNA polymerase were performed under the same conditions but with 1x V25 buffer instead of *Taq* buffer.

Twenty μl of the 100 μl reaction was separated on an agarose gel by electrophoresis and visualized by staining with ethidium bromide. Results are indicated by the relative amount of appropriately sized PCR product and all given in Table 1.

Table 1

Taq and *Pfu* DNA polymerases with CG7C7 template

Product	Primers	*Taq* buffer	V25 buffer
		Taq	*Pfu*
Fd	AB-19/AB-41	2+	+/-
CH1	MK-501/AB-41	2+	-
LC	AB-25/AB-26	+	-
polylinker	T3/T7	+	-

* The relative amounts of PCR product are indicated as a range of no product (-) to the most product (5+).

Results from this experiment indicate that only *Taq* DNA polymerase produced an appropriately sized PCR product with all of the primer pairs used. *Pfu* DNA polymerase produced either less or no product when compared to *Taq* DNA polymerase with all of the primer pairs.

The CH1 primers were designed for amplification of constant regions of the antibody heavy chain and would be expected to base pair match perfectly with their template. The T3 and T7 primers also base pair match perfectly with the pBluescript template.

Taq and *Pfu* DNA polymerases with PBL template

Taq DNA polymerase was used to amplify the LC (both *kappa* and *lambda*), CH1, CH2/CH3, and Fd regions of human PBL immunoglobulins. The $MgCl_2$ and dNTP concentrations were varied to try to increase the amount of PCR product generated. Total RNA was isolated from human PBLs using an RNA isolation kit (Stratagene). Five ug of total RNA was converted to cDNA in a first strand synthesis reaction using an oligo-dT primer for the LC, and MK-25 for the heavy chain. mRNA was annealed to the first strand primer at 65°C for 5 minutes followed by cooling at room temperature for 30 minutes. First strand reactions were performed in 1x first strand buffer [1x first strand buffer is 75 mM KCl; 50 mM tris-Cl, pH 8.3; 10 mM dithiothreitol; 3 mM $MgCl_2$; 1 Unit RNase Block II (Stratagene)], 375 μM each dNTP and 20 Units Moloney-Murine Leukemia Virus Reverse Transcriptase (M-MLVRT) (Stratagene)] and incubated at 42°C for 1 hour.

PCR primers used to amplify the *kappa* LC were AB-25 and AB-26, *lambda* LC were AB-92 and AB-28, CH2/CH3 were MK-25 and MK-26, Fd were AB-52 with AB-61, AB-62, AB-63, or AB-64 (Table 18). PCR reactions were at a primer concentration of 0.2 μM of each 5' and 3' primer in 1x *Taq* buffer (Table 17) with 2.5 units *Taq* DNA polymerase using 1/10 of cDNA prepared in the first strand reaction as the template. The *kappa* LC reaction was 2.0 mM $MgCl_2$ with 237.5 μM dNTPs, the *lambda* LC reaction was 1.8 mM $MgCl_2$ at 137.5 μM or 237.5 μM dNTPs, the Fd reactions were 1.9 mM $MgCl_2$ with 187.5 μM dNTPs, and the CH2/CH3 reactions were at 1.5 mM $MgCl_2$ with 137.5 μM dNTPs. Samples were denatured at 94°C for 5

minutes and annealed at 54°C for 5 minutes followed by PCR amplification at 72°C for 2.5 minutes, 93°C for 1 minute and 54°C for 1.5 minutes for 40 cycles. Samples were analyzed as described above. Results are indicated by the relative amount of appropriately sized PCR product and are given in Table 2.

Table 2

Taq and *Pfu* DNA polymerases with PBL template

PBL template						
		1.9 mM $MgCl_2$	1.8 mM $MgCl_2$		2.0 mM $MgCl_2$	
primers	product	187.5 µM dNTP	137.5 µM dNTP	237.5 µM dNTP	137.5 µM dNTP	237.5 µM dNTP
AB-61/AB-52	Fd	-				
AB-62/AB-52	Fd	-				
AB-63/AB-52	Fd	-				
AB-64/AB-52	Fd	-				
MK-25/MK-26	CH2/3	-	-	-	-	-
AB-25/AB-26	*kappa LC*					4+
AB-92/AB-28	*lambda LC*		+	4+	+	4+

Only the *kappa* and *lambda* LC reactions produced a specific PCR product. The 1.8 and 2.0 mM $MgCl_2$ with 137.5 µM dNTP *lambda* LC reactions produced only a very small amount of PCR product while the 1.8 and 2.0 mM $MgCl_2$ with 237.5 µM dNTPs produced a large amount of PCR product. This indicates that the amount of PCR product in this experiment is related to the dNTP and not the $MgCl_2$ concentration. No PCR product was generated with the Fd or CH2/CH3 primers under the conditions used.

EXAMPLE 2

PRIMER EXTENSION REACTIONS WITH 3' MISMATCHED PRIMERS

These experiments investigated the ability of *Taq* and *Pfu* DNA polymerases both together and in separate reactions to extend from primers which contain one or two 3' mismatches. The first experiment demonstrates that *Taq* DNA polymerase can only extend from a primer which matches at the 3' end under the conditions used (2.1 and 6.1 mM $MgCl_2$). The next experiment demonstrates that *Taq* and *Pfu* DNA polymerases used in the same reaction will extend from all the primers with 3' mismatches that were used from both hybridoma and PBL templates while neither polymerase alone was able to extend from all primers. The combination of both polymerases also resulted in more product in some of the samples.

This series of experiments suggest that *Taq* in *Taq* buffer will extend from a primer that is perfectly matched at the 3' end, in V25 buffer will extend from a primer that has one T which creates a mismatch at the 3' end of a primer, and in V25 buffer will not extend from a primer that has two Ts which create two mismatches at the 3' end of a primer. *Taq* and *Pfu* DNA polymerases in V25 buffer will extend from a primer that has two Ts which create a mismatch at the 3' end of a primer.

It was also speculated that *Taq* and *Pfu* DNA polymerases may complement each other in the reaction by polymerizing through A:T or G:C rich regions of a template where one enzyme could not polymerize well through both types of regions. The *Pfu* genome is 38% GC while the *Taq* genome is 66.8% GC.

Taq DNA polymerase with 9F12 template

The ability of *Taq* DNA polymerase to extend from primers with one or more mismatches at the 3' end was investigated. Nucleotide sequences of the 5' primers based on the AB-61 primer and used to amplify 9F12 cDNA are given in Table 12. The 3' primer in reactions which amplified the Fd portion of the human heavy chain was AB-76. The 3' primer in reactions which amplified the VH portion of the human heavy chain was MK-39. The light chain 5' VL primer was AB-25 and the 3' CL primer was AB-94.

9F12 cDNA template was generated by isolating total RNA from the hybridoma cells using an RNA isolation kit (Stratagene). mRNA is converted to cDNA in a first strand synthesis reaction using an oligo-dT primer for the light chain and AB-76 for the heavy chain. The first strand reactions was performed as described above except 200 units of Superscript (BRL; Gaithersburg, MD)] was substituted for M-MLVRT and the reactions were incubated at 42°C for 1 hour.

PCR reactions were at a primer concentration of 0.2 μM of each 5' and 3' primer in 1x *Taq* buffer (Table 17) with either 2.1 or 6.1 mM $MgCl^2$, 175 μM each dNTP and 2.5 units *Taq* DNA polymerase using 1/10 of the cDNA prepared in the first strand reaction as the template. Samples were denatured at 95°C for 5 minutes and annealed at 54°C for 5 minutes followed by PCR amplification at 72°C for 3 minutes, 93°C for 1.5 minutes and 54°C for 2.5 minutes for 40 cycles. Samples were analyzed as described above and the results are given in Table 3.

Table 3

Taq DNA polymerase with 9F12 template

		Taq in Taq buffer	
primers	product	2.1 mM $MgCl_2$	6.1 mM $MgCl_2$
AB-61/AB-76	Fd	2+	-
AB-714/AB-76	Fd	-	-
AB-715/AB-76	Fd	-	-
AB-716/AB-76	Fd	-	-
AB-717/AB-76	Fd	-	-
AB-61/MK-39	VH	2+	+

PCR products of the appropriate size were generated only when AB-61 was used as the 5' primer. None of the 3' mismatched primers generated a PCR product of the appropriate size although many unappropriately sized products were made. Fd and VH products were generated in the reaction containing 2.1 mM $MgCl_2$ while only the VH was produced in the reaction containing 6.1 mM $MgCl_2$.

These results suggested that *Taq* DNA polymerase was not able to extend from 3' mismatched primers under the conditions used and that increasing the $MgCl_2$ concentration did not result in either an increase in PCR product, the ability to extend from 3' mismatched primers, or generation of PCR products not made with 2.1 mM $MgCl_2$.

Taq and *Pfu* DNA polymerases with 9F12 and PBL templates

The next experiment investigated the effect of using *Pfu* and *Taq* DNA polymerases in the same PCR reaction to extend from primers with 3' matches and mismatches. The same primers were used (MK-205 which has the same 21 bp on the 3' end as AB-61 was substituted for AB-61) as described above to amplify Fd, VH, CH1, and LC from PBL and 9F12 cDNAs. PCR reactions were performed in 1x V25 buffer (Table 17) with 200 μM each dNTP and 2.5 units of both *Taq* and *Pfu* DNA polymerases or with *Pfu* DNA polymerase alone using cDNA generated as described above from 9F12 & PBL RNA as the template. Samples were denatured at 94°C for 5 minutes and annealed at 47°C for 5 minutes followed by PCR amplification at 71°C for 3 minutes, 92°C for 1 minute and 47°C for 2.5 minutes for 5 cycles and 71°C for 3 minutes, 92°C for 1 minute and 51°C for 2.5 minutes for 35 cycles. The lower annealing temperatures were used to try to improve primer:template annealing when mismatches occurred. Samples were analyzed as described above and the results are given in Tables 4 and 5.

Table 4

Taq and *Pfu* DNA polymerases with 9F12 and PBL templates

V25 buffer				
	9F12 template		PBL template	
	Pfu/Taq			*Pfu*
primers	Fd	VH	Fd	VH
AB-61/AB-76	5+	5+	+	+/-
AB-714/AB76	5+	5+	2+	+/-
AB-715/AB76	2+	5+	+/-	+/-
AB-716/AB76	5+	5+	2+	+/-
AB-717/AB76	5+	5+	+	+/-

Table 5

		9F12 templates		PBL templates	
		Pfu/Taq			*Pfu*
primers	product	Fd	VH	Fd	VH
MK-94/AB-76	CH1	5+		+/-	+/-
AB-25/AB-94	LC	+/-		+/-	+/-

An unexpected result in this experiment was that more amplification product was generated when *Taq* and *Pfu* DNA polymerases were used in the same PCR reaction in V25 buffer with both 9F12 and PBL cDNA as the template. Numerous previous experiments using identical first strand synthesis and PCR conditions with *Taq* DNA polymerase had resulted in only a very small amount of Fd product generated from the same PBL mRNA. A very small amount of VH and CH1 products were generated using *Pfu* DNA polymerase alone in V25 buffer with PBL cDNA as a template.

Significant amounts of both Fd and VH PCR products were generated with 9F12 cDNA as a template with all of the 5' primers, including the 3' mismatched primers (AB-714 to AB-717), when both *Taq* and *Pfu* DNA polymerases were used. This

contrasted with the previous experiment when *Taq* DNA polymerase was not able to extend from the 3' mismatched primers (AB-714 through AB-717) under the conditions used. These results suggested that *Pfu* DNA polymerase can remove 1-2 mismatched bases from the 3' end of the primer. This would enable either *Pfu* or *Taq* DNA polymerase to extend from the perfectly base paired primer:template.

Taq and *Pfu* DNA polymerases with 9F12 and PBL templates

The above experiment was repeated to verify the results. The same primers were used to amplify Fd, CH1, and LC using PBL and 9F12 cDNAs as templates. PCR reactions were performed in V25 buffer (Table 17) with 200 μM each dNTP and 2.5 units of both *Taq* and *Pfu* DNA polymerases either together in the same reaction and or in separate reactions. PCR conditions were the same as described above and the results are given in Table 5.

Table 6

Taq and *Pfu* DNA Polymerases with 9F12 and PBL Templates

		V25 buffer					
		9F12 template			PBL template		
primers	product	*Taq*	*Pfu*	*Pfu/Taq*	*Taq*	*Pfu*	*Pfu/Taq*
AB-61/AB-76	Fd	-	-	+	-	+	-
AB-714/AB-76	Fd	3+	-	4+	+	-	3+
AB-715/AB-76	Fd	-	-	2+	-	-	+
AB-716/AB-76	Fd	4+	-	4+	3+	+	+
AB-717/AB-76	Fd	2+	-	4+	+	-	3+
MK94/AB-76	CH1	4+	4+	4+	3+	-	-
AB-25/AB-94	LC	2+	-	-	+	-	-

PCR products generated with *Pfu* were Fd with MK-205 from PBL template and CH1 from 9F12 template. PCR products generated with *Taq* were Fd with AB-714, AB-716, AB-717, CH1, and LC with PBL template and Fd with AB-714, AB-716, AB-

717, CH1 and LC with 9F12 template. PCR products generated with *Pfu* and *Taq* were Fd with MK-205, AB-714, AB-717 and a very small amount with AB-715 and AB-716 from PBL template. PCR products generated with *Pfu* and *Taq* were Fd with AB-714, AB-715, AB-716, AB-717 and CH1 from 9F12 template.

The amount of PCR product varied, however, in general more product was generated with the 9F12 template than with the PBL template. This may be due to the heterogeneity of the PBL template when compared to the homogeneity of the 9F12 hybridoma template. When a homogeneous template is amplified and the strand is not completed, it may anneal to its template in a subsequent round of amplification and be extended to full length. When a heterogeneous template is amplified and the strand is not complete, the chances of it annealing to its original template in a subsequent round of amplification will be related to the heterogeneity of the templates present but will be less than that of a homogeneous template. This may help explain why homogeneous templates produce more PCR product than heterogenous templates.

In this experiment *Taq* DNA polymerase was able to extend from the 3' mismatched primers which contain only a single mismatched base (AB-714, AB-716, and AB-717) but not one that has two mismatched bases (AB-715) with both the PBL and 9F12 cDNA templates. In a previous experiment, *Taq* DNA polymerase was not able to do this, however, these results are consistent with the results of Kwok, et al. (1990, supra). Both AB-715 and AB-717 contain 2 dTTPs at the 3' end of the primer but in different positions. AB-715 replaces the last two dGTPs on the 3' end and AB-717 replaces the last dGTP and adds a dTTP to the 3' end. Because the corresponding base in the template is not known, the 3' dTTP in AB-717 may not create a mismatch with the template.

The V25 buffer used in this experiment had been made incorrectly and was 5 mM KCl instead of 10 mM KCl. KCl concentration has been shown to effect processivity and/or activity of *Taq*. This was corrected for all future experiments.

EXAMPLE 3

EFFECT OF DIFFERENT RATIOS OF *TAQ* AND *PFU* DNA POLYMERASES ON EXTENSION FROM 3' MISMATCHED PRIMERS

This experiment investigated different ratios of *Taq* and *Pfu* DNA polymerases and template concentrations when amplifying from perfectly matched and 3' mismatched primers. Plasmid DNA which encoded an anti-tetanus toxoid immunoglobulin fragment (*kappa* light chain and Fd portion of the heavy chain) was used as the template (Mullinax et al. 1990, supra). Although optimal polymerase ratios and template concentrations were not identified in these experiments, additional experimentation would need to be done before concluding that they did not have an effect.

EFFECT OF *PFU* DNA POLYMERASE RATIO ON EXTENSION FROM 3' MISMATCHED PRIMERS

Five different ratios of *Taq* and *Pfu* DNA polymerases were used in PCR reactions with a combined total of 2.5 units per reaction. The ratios were 9:1, 7:3, 5:5, 3:7, and 1:9 of *Taq:Pfu*. The Fd primers were AB-61, AB-715, and AB-717 in V25 buffer. Anti-tetanus toxoid plasmid DNA encoding a *kappa* LC and Fd was used as the template. Samples were denatured at 95°C for 7 minutes and annealed at 40°C for 7-10 minutes followed by PCR amplification at 72°C for 2 minutes, 95°C for 1 minute and 50°C for 2 minutes for 30 cycles. Samples were analyzed as described above and results are given below.

Table 7

EFFECT OF DIFFERENT RATIOS OF *TAQ* AND *PFU* DNA POLYMERASES ON EXTENSION FROM 3' MISMATCHED PRIMERS

		ratio of *Taq:Pfu* DNA polymerases						
primers	product	10:0	9:1	7:3	5:5	3:7	1:9	0:10
AB-61/AB-76	Fd	4+	5+	5+	5+	5+	4+	4+
AB-715/AB-76	Fd	-	5+	5+	5+	5+	2+	5+
AB-717/AB-76	Fd	2+	5+	5+	5+	5+	5+	5+

All PCR reactions generated a significant amount of PCR product with little variation except the reactions with AB-715 and AB-717 with *Taq* DNA polymerase alone and the reaction with *Taq*:*Pfu* at 1:9 with AB-715. All of the reactions which combine both *Taq* and *Pfu* DNA polymerases or contain *Pfu* alone produced a significant amount of product. *Taq* DNA polymerase alone not would be expected to extend efficiently from the 3' mismatched primers.

Template concentration can affect the amplification efficiency and may explain there is little difference in the amount of PCR product in the different samples when the ratio of DNA polymerases is varied. In the next experiment, three different template concentrations were used to try to determine the effect of template concentration.

EFFECT OF TEMPLATE CONCENTRATION

Template concentrations were 100, 50, and 10 nanograms per reaction with either 2.5 units of *Taq* or *Pfu* DNA polymerase in the reaction in V25 buffer. The Fd primers were AB-61, AB-715, and AB-717 in V25 buffer. Samples were denatured at 95°C for 7 minutes and annealed at 40°C for 7-10 minutes followed by PCR amplification at 72°C for 2 minutes, 95°C for 1 minute and 50°C for 2 minutes for 30 cycles. Samples were analyzed as described above and results are given in Table 8.

Table 8

EFFECT OF TEMPLATE CONCENTRATION

		template concentration					
		100 ng		50 ng		10 ng	
primers	product	*Taq*	*Pfu*	*Taq*	*Pfu*	*Taq*	*Pfu*
AB-61/AB-76	Fd	5+	5+	5+	5+	5+	5+
AB-715/AB-76	Fd	5+	5+	5+	5+	5+	5+
AB-717/AB-76	Fd	5+	5+	5+	5+	5+	2+

Varying the template concentration did not seem to have an effect on the amount of PCR template generated except in the 10 ng template sample with *Pfu* DNA

polymerase alone with AB-717. The results seen previously with AB-715 where there was no amplification with *Taq* DNA polymerase alone was not reproduced and the experimenter indicated that an error could have been made. The experiment with AB-715 alone was repeated.

This experiment repeated the experiment described above using just the AB-715 primer under the same conditions. Samples were analyzed and described above and results are given in Table 9.

Table 9

EFFECT OF TEMPLATE CONCENTRATION

		template concentration					
		100 ng		50 ng		10 ng	
primer	product	*Taq*	*Pfu*	*Taq*	*Pfu*	*Taq*	*Pfu*
AB-715/AB-76	Fd	4+	3+	3+	2+	1+	5+

The amount of PCR product generated correlated with template concentration in the reactions with *Taq* DNA polymerase alone and may indicate that the template concentration affects amplification efficiency from mismatched primers. No consistent effect could be correlated with *Pfu* DNA polymerase.

EXAMPLE 4

EFFECT OF ANNEALING TEMPERATURE ON EXTENSION FROM 3' MISMATCHED PRIMERS

The next experiment compared *Taq* and *Taq* with *Pfu* in *Taq* and V25 buffers with mismatched primers at different annealing temperatures. This experiment demonstrates that more PCR product is generated from a perfectly matched primer at a lower annealing temperature than at a higher temperature. No product was produced with the primer with 2 mismatches under any of the conditions used.

Effect of annealing temperatures of extension from 3' matched and mismatched primers

Taq and *Taq* with *Pfu* in *Taq* and V25 buffers with mismatched primers were extended at different annealing temperatures. The Fd primers were AB-61 and AB-715. cDNA templates were prepared from PBLs and 9F12. Samples were denatured at 95°C for 5 minutes and annealed at either 48°C or 52°C for 5 minutes followed by PCR amplification at 71°C for 3 minutes, 92°C for 1 minute and 48°C or 52°C for 2.5 minutes for 5 cycles and 71°C for 3 minutes, 92°C for 1 minute and 54°C for 2.5 minutes for 40 cycles. The lower annealing temperatures were used determine the effect the lower temperature had on primer:template annealing when mismatches occurred. Samples were analyzed as previously described and results are given in Table 10.

Table 10

Effect of Annealing Temperatures on Extension From 3' Matched and Mismatched Primers

		annealing at 48°C (x5 cycles) and 52°C (x35 cycles)							
		9F12 template				PBL template			
		Taq buffer		V25 buffer		*Taq* buffer		V25 buffer	
primers	product	*Taq*	*Pfu/ Taq*	*Taq*	*Pfu/ Taq*	*Taq*	*Pfu/ Taq*	*Taq*	*Pfu/ Taq*
AB-61/AB-76	Fd	4+	4+	4+	4+	+/-	+	-	+/-
AB-715/AB-76	Fd	-	-	-	-	-	-	-	-

		annealing at 54°C (x40 cycles)							
		9F12 template				PBL template			
		Taq buffer		V25 buffer		*Taq* buffer		V25 buffer	
primers	product	*Taq*	*Pfu/ Taq*	*Taq*	*Pfu/ Taq*	*Taq*	*Pfu/ Taq*	*Taq*	*Pfu/ Taq*
AB-61/AB-76	Fd	4+	4+	2+	+	-	-	-	-
AB-715/AB-76	Fd	-	-	-	-	-	-	-	-

PCR products generated with *Pfu* and *Taq* in *Taq* or V25 buffers were a very small amount of Fd using AB-61 with the PBL template at the lower and not the higher annealing temperature. PCR products generated with *Taq* with and without *Pfu* in *Taq* or V25 buffer using AB-61 with the 9F12 template resulted in more product at the lower rather than the higher annealing temperature. No PCR products were generated with the AB-715 primer in any samples.

Results indicated that none of the polymerases or buffer combinations tested extended from the AB-715 primer. AB-61 and AB-715 differ by the deletion of two dGTPs and the addition of two dTTPs at the 3' end (Table 13). The lower annealing temperatures did produce either the presence of or an increase in the amount of PCR product generated.

EXAMPLE 5

PCR REACTIONS USING PRIMERS WITHOUT 3' MISMATCHES

The object of the experiments with the 3' mismatched primers was to determine PCR conditions would result in a sufficient amount of high fidelity PCR product to enable cloning of the encoded immunoglobulin fragments. Therefore, the original PCR primers designed to amplify all human heavy chain genes were now used in the PCR reactions with *Taq* and *Pfu* DNA polymerases separately and in the same reaction. Results indicate that there was no significant difference in the amount of PCR product generated when either *Taq* or *Taq* and *Pfu* DNA polymerases were used in these experiments.

Taq and *Pfu* DNA polymerases with 9F12 and PBL templates

The first strand synthesis and PCR reactions were performed as described above under PCR REACTIONS WITH 3' MISMATCHED PRIMERS - *Taq* and *Pfu* DNA polymerases with 9F12 and PBL templates with the following change. AB-61, AB-62, AB-63, and AB-64 (Table 18) were used as the 5' VH primers. Samples were analyzed as described above and results are given in Table 11.

Table 11

Taq and *Pfu* DNA Polymerases With 9F12 and PBL Templates

		V25 buffer					
		9F12 template			PBL template		
primers	product	*Taq*	*Pfu*	*Pfu/ Taq*	*Taq*	*Pfu*	*Pfu/ Taq*
AB-61/AB-76	Fd	4+	-	-	4+	+/-	4+
AB-62/AB-76	Fd	4+	-	4+	4+	+/-	4+
AB-63/AB-76	Fd	4+	+	4+	+/-	-	-
AB-64/AB-76	Fd	4+	-	4+	2+	-	2+
MK-94/AB-76	CH1	4+	3+	4+	2+	-	3+
AB-25/AB-94	LC	2+	-	3+	-	-	+

PCR products generated with *Pfu* were a very small amount of Fd with AB-61 and AB-62 from PBL template and Fd with AB-63 and CH1 from 9F12 template. PCR products generated with *Taq* were Fd with all 5' primers and CH1 PBL and 9F12 templates and LC with 9F12 template. PCR products generated with *Pfu* and *Taq* were Fd with AB-61, AB-62, AB-64, CH1, and LC from PBL template. PCR products generated with *Pfu* and *Taq* were Fd with AB-62, AB-63, AB-64, CH1, and LC from 9F12 template. In general, the results with the PBL template had a lot of failure products and there was significantly more product with the 9F12 template.

The inability of *Pfu* DNA polymerase to extend from any of the 5' primers other than AB-63 may be due to internal mismatches with 9F12 as the template and not be due to the 3' primer:template mismatches.

EXAMPLE 6

The effect of a combination of four different polymerases in seven different buffers was tested on three different templates. The desired effects were an increase in the amount of PCR product generated and/or an increased specificity of amplification.

No effect was observed between using a combination of four polymerases and *Taq* DNA polymerase in the buffers tested.

Seven different templates with eight different primer sets were used in this experiment. The genomic DNAs were isolated from human, Epstein Barr virus, *Escherichia coli*, and transgenic and normal mouse. Plasmid DNAs were pBluescript II and pBluescript containing the light chain and Fd of an anti-tetanus toxoid immunoglobulin (Mullinax et al., 1990, supra). Primer sequences and their respective DNA templates are given in Table 16. The thermostable polymerases used in this experiment were *Taq*, Exo$^+$ and Exo$^-$ *Pfu*, and ES-4 DNA polymerases (Stratagene; La Jolla, California) at final concentrations of 2.5, 1.25, 1.25, and 0.125 units per reaction, respectively. PCR reactions were at a primer concentration of 0.2 μM of each 5' and 3' primer in 1x *Taq* buffer, 1x *Pfu* #1, or 1x *Pfu* #3 (Table 17) with 200 μM each dNTP. An additional reaction with *Taq* DNA polymerase in 1x *Taq* buffer was also performed. Samples were denatured at 95°C for 5 minutes and annealed at 40°C for 5 minutes followed by PCR amplification at 95°C for 0.5 minutes, 40°C for 1.5 minutes and 68°C for 3 minutes for 25 cycles. Samples were analyzed as previously described and results are not given.

Results indicated that no significant increase in the amount of PCR product generated or an increase in specificity was observed when comparing *Taq* DNA polymerase in *Taq* buffer and a combination of polymerases in the three different buffers tested. Results seemed to correlate with the buffer used and not the polymerase.

EXAMPLE 7

TAQ AND *PFU* DNA POLYMERASES IN SPLICE OVERLAP EXTENSION REACTIONS TO GENERATE CLONING VECTORS

A series of lambda-based vectors were constructed to express immunoglobulin fragments on the surface of M13 phage. The example described below describes the construction of one of these vectors. *Pfu* and *Taq* DNA polymerases in combination were used to construct this vector because of the high processivity of *Taq* DNA polymerase and the high fidelity of *Pfu* DNA polymerase. The nucleotide sequence of the cpVIII expression vector matched the expected nucleotide sequence.

cpVIII expression vector construction

Oligonucleotide primers used to construct the IZ H-8 (-1 to 50) vector with the cpVIII protein domain were designed to encode the following: an Xho I restriction site, stop codons, nucleotide spacer sequences, an Xba I site that generates an amber codon, amino acids -1 to 50 of cpVIII, stop codons, and a Spe I restriction site.

Nucleotide sequences of the primers used to amplify cpVIII from M13 Phagescript DNA (Stratagene; La Jolla, California) using the 5' and 3' cpVIII primers are given in Table 19. All PCR reactions were at a primer concentration of 0.2 μM of each 5' and 3' primer in 1x V25 buffer with 200 μM each dNTP and 2.5 units each *Taq* and *Pfu* DNA polymerases. Samples were denatured at 95°C for 5 minutes and annealed at 54°C for 5 minutes followed by PCR amplification at 74°C for 3 minutes, 93°C for 1.5 minutes and 54°C for 2.5 minutes for 30 cycles.

The cpVIII encoding PCR product was restriction digested with Xho I and Spe I and ligated into the IZ H vector (Stratacyte; La Jolla, California; Huse, W.D., L. Sastry, S.A. Iverson, A.S. Kang, M. Alting-Mees, D.R. Burton, S.J. Benkovic, and R.A. Lerner. Generation of a large combinatorial library of the immunoglobulin repertoire in phage lambda. 1989. *Science*. **246**:1275-1281) which had previously been restriction digested with Xho I and Spe I. The ligation products were packaged with Gigapack II® Gold (Stratagene; La Jolla, California) and plated with XL1-Blue host cells. The desired construct was identified by PCR amplification of isolated phage cores with the 5' and 3' cpVIII cloning primers, converted to the plasmid format by *in vivo* excision with VCS M13 helper phage (Short, J., J.M Fernandez, J.A. Sorge, and W.D. Huse, Lambda ZAP: a bacteriophage lambda vector with *in vitro* excision proprites, 1988, *Nucleic Acids Res.* **16**:7583-7600) and the nucleotide sequence was determined by the dideoxy method. The nucleotides was sequence as expected.

Table 12

HUMAN HEAVY CHAIN PCR PRIMERS

WITH MATCHED AND 3' MISMATCHES VH PRIMERS

primer	description	nucleotide sequence (5' to 3')
AB-205	5' VH	GTCCTGTCCGAGGTGCAGCTGCTCGAGTCTGG (SEQ ID NO: 1)
AB-61	5' VH	AGGTGCAGCTGCTCGAGTCTGG (SEQ ID NO: 2)
AB-714	5' VH	AGGTGCAGCTGCTCGAGTCTG**T** (SEQ ID NO: 3)
AB-715	5' VH	AGGTGCAGCTGCTCGAGTCT**TT** (SEQ ID NO: 4)
AB-716	5' VH	AGGTGCAGCTGCTCGAGTCTGG**T** (SEQ ID NO: 5)
AB-717	5' VH	AGGTGCAGCTGCTCGAGTCTG**TT** (SEQ ID NO: 6)
AB-76	3' CH1	AGCATCACTAGTACAAGATTTGGGCTC (SEQ ID NO: 7)
AB-52	3' CH1	CGGGAGATCATGAGGGTGTCCTT (SEQ ID NO: 8)
MK-39	3' VH	ATATACTAGTGAGACAGTGACCAGGGTTCC TTGGCCCCA (SEQ ID NO: 9)

Table 13

HUMAN HEAVY AND LIGHT CHAIN PCR PRIMERS

primer	description	nucleotide sequence (5' to 3')
AB-205	5' VH	GTCCTGTCCGAGGTGCAGCTGCTCGAGTCTG G (SEQ ID NO: 1)
AB-61	5' VH	AGGTGCAGCTGCTCGAGTCTGG (SEQ ID NO: 2)
AB-714	5' VH	AGGTGCAGCTGCTCGAGTCTG**T** (SEQ ID NO: 3)
AB-715	5' VH	AGGTGCAGCTGCTCGAGTCT**TT** (SEQ ID NO: 4)
AB-716	5' VH	AGGTGCAGCTGCTCGAGTCTGG**T** (SEQ ID NO: 5)
AB-717	5' VH	AGGTGCAGCTGCTCGAGTCTG**TT** (SEQ ID NO: 6)
AB-76	3' CH1	AGCATCACTAGTACAAGATTTGGGCTC (SEQ ID NO: 7)
AB-52	3' CH1	CGGGAGATCATGAGGGTGTCCTT (SEQ ID NO: 8)
MK-39	3' VH	ATATACTAGTGAGACAGTGACCAGGGTTCC TTGGCCCCA (SEQ ID NO: 9)
AB-25	5' VL	GTGCCAGATGTGAGCTCGTGATGACCCAGT CTCCA (SEQ ID NO: 10)
AB-94	3' CL	TCCTTCTAGATTACTAACACTCTCCCCTGTT GAAGCTCTTTGTGACGGGCGAACTC (SEQ ID NO: 11)
MK-94	5' CH1	GTCTCACTAGTCTCCACCAAGGGCCCATCGG TC (SEQ ID NO: 12)
AB-58	3' CH2	CGGGAGATCATGAGGGTGTCCTT (SEQ ID NO: 13)
MK-25	5' CH2	CTCAGTATGGTGGTTGTGC (SEQ ID NO: 14)

primer	description	nucleotide sequence (5' to 3')
MK-26	3' CH3	CCGGAATTCTTATCATTTACCCGGAGA (SEQ ID NO: 15)

* nucleotides in bold indicate where dTTP was introduced into the AB-61 primer

Table 14

pBluescript PCR primers

description	nucleotide sequence (5' to 3')
T3	ATTAACCCTCACTAAAG (SEQ ID NO: 16)
T7	AATACGACTCACTATAG (SEQ ID NO: 17)

Table 15

MOUSE HEAVY AND LIGHT CHAIN PCR PRIMERS

primer	description	nucleotide sequence (5' to 3')
AB-25	5' VL *kappa*	GTGCCAGATGTGAGCTCGTGATGACCC AGTCTCCA (SEQ ID NO: 10)
AB-26	3' CL *kappa*	TCCTTCTAGATTACTAACACTCTCCCCT GTTGAA (SEQ ID NO: 18)
AB-28	5' VL *lambda*	CTGCACAGGGTCCTGGGCCGAGCTCGT GGTGACTCAG (SEQ ID NO: 19)
AB-92	3' VL *lambda*	GCATTCTAGACTATTAACATTCTGTAGG GGC (SEQ ID NO: 20)
AB-19	5' VH	AGGTCCAGCTGCTCGAGTCTGG (SEQ ID NO: 21)
AB-41	3' CH1	AGGCTTACTAGTACAATCCCTGGGCAC AAT (SEQ ID NO: 22)
MK-501	3' VH	CCGTTACTAGTAGCCAAAACGACACCC CCATCTGTC (SEQ ID NO: 23)

Table 16

DNA TEMPLATES AND PCR PRIMER SEQUENCES

template description	primer description	nucleotide sequence (5' to 3')
human	5' Gaucher's disease	CCTGAGGGCTCCCAGAGAGTGG (SEQ ID NO: 24)
human	3' Gaucher's disease	GGTTTAGCACGACCACAACAGC (SEQ ID NO: 25)
pBluescript KS+	5' multiple cloning site	ATTAACCCTCACTAAA (SEQ ID NO: 26)
pBluescript KS+	3' multiple cloning site	AATACGACTCACTATAG (SEQ ID NO: 27)
Epstein Barr virus	5' nuc antigen gene	GGCTGGTGTCACCTGTGTTA (SEQ ID NO: 28)
Epstein Barr virus	3' nuc antigen gene	CCTTAGGAGGAACAAGTCCC (SEQ ID NO: 29)
E. coli	5' RNase H gene	CTTGAAGATCTATGCTTAAACAGGTAG (SEQ ID NO: 30)
E. coli	3' RNase H gene	CATGTGAATTCTTAAACTTCAACTTGG (SEQ ID NO: 31)
transgenic mouse	5' lambda lacZ insert	GGTGGCGACGACTCCTGGAGCCC (SEQ ID NO: 32)
transgenic mouse	3' lambda lacZ insert	GACAGTCACTCCGGCCCGTGCGG (SEQ ID NO: 33)
human	5' fucosidase gene	AAGCTTCAGGAAAACAGTGAGCAGCGCCTC (SEQ ID NO: 34)
human	3' fucosidase gene	AGTCAGGTATCTTTGACAGT (SEQ ID NO: 35)

nontransgenic mouse	5' B-adrenergic receptor	GGAATTCGTAACAGCACTTA CGGTAGC (SEQ ID NO: 36)
nontransgenic mouse	3' B-adrenergic receptor	AGCACTCATAAGTGACACCC (SEQ ID NO: 37)
transgenic mouse	5' lambda lacI insert	CATAGCGAATTCGCAAAACC TTTCGCGGTATGG (SEQ ID NO: 38)
transgenic mouse	3' lambda lacI insert	ACTACGGAATTCCACGGAAA ATGCCGCTCATCC (SEQ ID NO: 39)

Table 17

10x PCR BUFFER COMPONENTS

component	*Taq*	V25	*Pfu* #1	*Pfu* #3
KCl	500 mM	100 mM	100 mM	100 mM
$(NH_4)_2SO_4$		60 mM	60 mM	100 mM
tris-Cl, pH 8.8	100 mM			
tris-Cl, pH 8.2		200 mM	200 mM	
tris-Cl, pH 8.5				200 mM
MgCl2	15 mM	15 mM	20 mM	15 mM
MgSO4				20 mM
Triton X-100		1%(v/v)	1%(v/v)	1%(v/v)
bovine serum albumin		100 µg/ml	100 µg/ml	1 mg/ml
gelatin	0.01% (w/v)			

Table 18

HUMAN VH PCR PRIMERS

primer	description	nucleotide sequence (5' to 3')
AB-61	5' VH	AGGTGCAGCTGCTCGAGTCTGG (SEQ ID NO: 2)
AB-62	5' VH	AGGTGCAGCTGCTCGAGTCGGG (SEQ ID NO: 40)
AB-63	5' VH	AGGTGCAACTGCTCGAGTCTGG (SEQ ID NO: 41)
AB-64	5' VH	AGGTGCAACTGCTCGAGTCGGG (SEQ ID NO: 42)

Table 19

OLIGONUCLEOTIDE PRIMERS USED TO CONSTRUCT

THE IZ H-8 (-1 to 50) VECTOR

5' cpVIII	TTGACTCGAGTAATCTGAGCTAAAGTCTAGAGCGCTGAGGGTG ACGATCC (SEQ ID NO: 43)
3' cpVIII	ATGGCAACTAGTTATCAGCTTGCTTTCGAGG (SEQ ID NO: 44)

EQUIVALENTS

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. Indeed, various modifications of the above-described modes for carrying out the invention which are obvious to those skilled in the field of molecular biology or related fields are intended to be within the scope of the following claims.